THYROID HORMONE ANALYSIS BY MASS SPECTROMETRY

Field of the Invention

[0001] The present invention combines the fields of hormone analysis and mass spectrometry. In particular the invention relates to analyzing thyroid hormones using mass spectrometry.

Background of the Invention

[0002] Hormones are biological messengers. They are synthesized by specific tissues (glands) and are secreted into the blood. The blood carries them to target cells where they act to alter the activities of the target cells.

[0003] Hormones are chemically diverse, and are generally categorized into three main groups: (1) small molecules derived from amino acids, for example thyroxine, (2) polypeptides or proteins, for example insulin and thyroid-stimulating hormone, and (3) molecules derived from cholesterol, for example steroids.

[0004] An important class of hormone is the thyroid hormones. Examples of thyroid hormones are thyroxine (T4), free thryoxine (FT4) and triiodothyronine (T3). T4 and T3 enter cells and bind to intracellular receptors where they increase the metabolic capabilities of the cell by increasing mitochondria and mitochondrial enzymes. T4 and T3 are important in regulating a number of biological processes, including growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis and fetal neurodevelopment. Synthesis of all circulating T4 and a small percentage of circulating T3 occurs on thyroglobulin molecules located within the thyroid. The bulk of the T3 present in the blood is produced enzymatically via monodeiodination of T4 by specific intracellular deiodinases – enzymes present in the follicular cells and the cells of target tissues [1]. In serum drawn from healthy human subjects, total T4 is present at about 60-fold higher concentration than total T3. T4 acts as a prohormone, as the reservoir for the production of T3, the active hormone. The metabolic activity associated with thyroid hormone (TH) is initiated by T3 binding to specific nuclear receptors within

target cells. Thyroid hormone concentrations in blood are essential tests for the assessment of thyroid function.

[0005] Steroids make up another important class of hormones. Examples of steroid hormones include estrogens, progesterone and testosterone. Estrogen is the name of a group of hormones of which there are three principle forms, estrone, estradiol and estriol. Estrogens and progesterone cause the development of the female secondary sexual characteristics and develop and maintain the reproductive function. Testosterone develops and maintains the male secondary sex characteristics, promotes growth and formation of sperm. Steroids enter target cells and bind to intracellular receptors and then cause the production of mRNA coding for proteins that manifest the changes induced by steroids.

[0006] The accurate analysis and quantification of hormones is becoming more important. For example, estrogen and estrogen like compounds are playing an ever-increasing role in today's society through hormone replacement therapy. Also, the analysis and quantification of estrogen and estrogen-like compounds helps in the management of estrogen-related diseases, like breast cancer. In addition, the accurate analysis and quantification of T4 and T3 is an issue recognized by those skilled in the art. The presence of circulating iodothyronine-binding autoantibodies that interfere with total T4 and T3 radioimmunoassays ("RIAs") is a known phenomenon [2], [3], [4]. These autoantibodies may give falsely high, or falsely low values of thyroid hormone measurements depending on the assay separation method used, and are often in discordance with the clinical features [2], [3], [4]. Direct serum free T4 and T3 (FT4 and FT3) measurements are a way to compensate for such abnormal binding. However, technically, it is difficult to measure the free hormone concentrations since these are so low. It is easier to measure the total (free and protein-bound) thyroid hormone concentrations; total hormone concentrations are measured at nanomolar levels whereas free hormone concentrations are measured in the picomole range and to be valid, must be free from interference by the much higher total hormone concentrations.

[0007] Presently, the common methods of hormone analysis use immunoassay techniques. Table 1 lists the common hormones and the current methods for their analysis.

[0008] For example, estriol is analyzed by a radioimmunoassay utilizing radiolabelled antigen (iodine 125) in competition with unlabelled estriol in the sample, for a known amount of antibody. The assay is read using a gamma counter.

[0009] Androstenedione is analyzed using an enzyme immunoassay comprising horseradish peroxidase. Unlabeled antigen in the sample is in competition with enzyme labeled antigen for a fixed number of antibody binding sites. The assay is read using a microtitre plate enzyme immunoassay reader.

[00010] Several hormones are currently analyzed using a chemiluminescent immunoassay. For example, progesterone, testosterone, cortisol and T3 are analyzed using this method. The assay utilizes an assay-specific antibody-coated bead. The assay is read using a photon counter.

[00011] However, the current immunoassays are disadvantageous for the following reasons:

(1) Immunoassays are specific to one hormone, therefore every hormone must be analyzed separately.

(2) Numerous kits must be purchased and procedures must be learned for each hormone being analyzed.

(3) Various instruments to read the results from the immunoassays must be purchased. For example, the analysis of estriol and progesterone from a sample requires both a gamma counter and a photon counter.

(4) The kits for the assays can be expensive.

(5) The current immunoassays lack specificity and may show approximately 15 fold difference in results using kits from different manufacturers [5].

(6) The procedures involve many steps and can take a significant amount of time.

(7) In the case of a radioimmunoassay, precautions are necessary because of the radioisotopes involved.

[00012] Immunoassays are notoriously unreliable with more and more literature published supporting their lack of specificity [6-13]. Table 2 shows the major differences reported by the College of American Pathologists program for proficiency testing of thyroid hormones that clearly illustrates the difference in specificity of the various antibodies used. For example, Table 2 shows mean results between different methods reported in the College of American Pathologists Proficiency Testing (CAP PT) Program can vary by a factor of approximately 2. Some factors such as pregnancy, estrogen therapy or genetic abnormalities in protein binding have also reportedly made Immunoassay methods for T4 and T3 diagnostically unreliable [2], [3], [14], [15]. Currently serum total T4 (TT4) and total serum T3 (TT3) concentrations are most commonly measured by immunoassay methods. Recently some reports of quantitative measurement of T4 and T3 by high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) were published [16-20]. All those methods required extraction, derivatization and even prior chromatographic separation that are very time-consuming [21], [22].

[00013] More recently, hormones have been analysed and quantified by mass spectrometry. However, there are several disadvantages to these methods.

[00014] For example, a method of analyzing urinary testosterone and dihydrotestosterone glucuronides using electrospray tandem mass spectrometry has been described [23]. The method involves a complex system employing high

testosterone and the limit of quantification was 10 ug L^{-1} for dihydrotestosterone and (v) the method is complex.

[00015] Another publication discloses a method for the determination of estradiol in bovine plasma by an ion trap gas chromatography-tandem mass spectrometry technique [24]. The shortcomings include the following: (i) only one analyte was analyzed, (ii) 4 ml of plasma was required for the analysis of one analyte, (iii) the limit of detection was 5 pg ml^{-1}, and (iv) derivation was required because the method employs gas chromatography.

[00016] A method for analysis of 17-hydroxyprogesterone by HPLC electrospray ionization tandem mass spectrometry from dried blood spots has also been described [25]. However, this method analyses only one analyte at a time, and requires liquid-liquid extraction, which is laborious and time consuming, with sample extraction alone taking 50 minutes to complete.

[00017] Finally, a gas chromatography mass spectrometry method to analyze the production rates of testosterone and dihydrosterone has been disclosed [26].

TABLE 1: METHODS AND INSTRUMENTS FOR STEROID AND THYROID HORMONES [1].

ANALYTE	Percentage of Use	Instrument	METHOD
Androstenedione	35%	DSL solid	EIA
11-Deoxycortisol	50%	ICN Immuchem DA	RIA
DHEA Sulfate	39%	DPC Immulite	ECIA
Estradiol	16%	Bayer ADVIA Centaur	FIA
Estriol, unconjugated	25%	DSL liquid	RIA
Estriol, Total	50%	DPC Coat-a-Count	RIA
17-Hydroxyprogesterone	51%	DPC Coat-a-Count	RIA
Progesterone	23%	Bayer ADVIA Centaur	CIA
Testosterone	29%	Bayer ADVIA Centaur	CIA
Testosterone, Free	65%	DPC Coat-a-Count	RIA
Aldosterone	76%	DPC Coat-a-Count	RIA
Cortisol	25%	Bayer ADVIA Centaur	CIA
T3	29%	Abbott Axsym	FPIA
T3, Free	31%	Bayer ADVIA Centaur	CIA
T4	30%	Abbott Axsym	FPIA
T4, Free	34%	Abbott Axsym	FPIA

RIA: Radioimmunoassay
EIA: Enzyme Linked Immunoassay
FPIA: Fluorescence Polarization Immunoassay

Table 2: Problems with Immunoassays: Data acquired from CAP PT Program 2003

Analyte	Mean CAP Result for Method Giving Lowest Value	Mean CAP Result for Method Giving Highest Value
Triiodothyronine (ng/dL)	108.5	190.2
	364.8	610.1
Thyroxine (ug/dL)	5.64	10.09
	1.64	3.65
	8.73	13.12

Table 2: Problems with Immunoassays: Data acquired for samples from the CAP PT Program 2003.

Summary of the Invention

[00018] The invention provides a fast and accurate method of hormone analysis and quantification using a mass spectrometer.

[00019] A plurality of hormones can be analyzed simultaneously or sequentially. The procedure allows for as little as 100 μL of a sample to be analyzed. In addition, minimal sample preparation time is required.

[00020] The invention permits the analysis of hormones in a number of complex matrices as they might be found in nature, e.g. the human body. For, example, hormone analysis can be performed on samples of blood, saliva, serum, plasma and urine.

[00021] There are several advantages to this invention:

(1) It provides a total and specific analysis for hormones in a sample. The present method allows for the analysis of many hormones simultaneously or sequentially.

(2) The procedure does not require an immunoprecipitation reaction. The majority of other methods for hormone analysis required an immunoassay. Immunoassays are expensive, specific to a particular analyte and involve several steps.

(3) The present invention requires minimal sample preparation time. For example, preparing a sample for hormone analysis can be done within 6 minutes.

(4) The procedure does not require a large sample size. A plasma or serum sample can be as small as 100 μL for thyroid hormones. The current methods for hormone analysis that utilize mass spectrometry require 4-15 mL of plasma.

(5) The invention uses simple preparation techniques that are easy to use and highly reproducible.

(6) The invention permits analysis to be performed on a variety of sample types.

(7) The invention permits the analysis of hormones in a sample of saliva or urine which permits simple sample acquisition and the remote submission of samples to a clinic for analysis. In previous other clinical methods, samples are taken by invasive means directly from the patient in a clinic.

(8) The analysis by mass spectrometry is highly accurate. In addition, the procedure of the present invention is highly reproducible.

(9) The invention permits the analysis of a wide range of hormone concentrations. In addition, the limit of detection can be fairly low.

[00022] Accordingly, there is provided a use for a mass spectrometer for simultaneously or sequentially analyzing a sample for a plurality of hormones in a fast, simple and accurate way. The sample may be, for example, serum, plasma, urine or saliva.

[00023] There is also provided a system for the fast, simple and accurate analysis of a plurality of hormones comprising: reagents for the preparation of the sample, reagents to perform the analysis on a mass spectrometer, and a mass spectrometer to perform the analysis.

[00024] There is also provided a kit, comprising the various reagents required for simultaneously or sequentially analyzing, within a sample, a plurality of hormones, including steroid hormones, thyroid hormones and other hormones. The kit may include a standard solution of the hormones of interest, compounds as internal standards, mobile phase solutions, methods and tools for separating hormones from samples, for example HPLC columns, and quality control specimens.

[00025] There is also provided a method for the simultaneous or sequential analysis of one or more hormones comprising ionizing the hormones and analyzing the hormones by mass spectrometry.

[00026] Accordingly, there is also provided a method for the simultaneous or sequential analysis of one or more hormones comprising: obtaining a sample containing or suspected of containing one or more hormones, removing proteins from the sample,

separating the hormones from the sample, ionizing the hormones and analyzing the hormones in a mass spectrometer.

[00027] Accordingly, there is also provided a method for the analysis of one or more thyroid hormones comprising: obtaining a sample containing or suspected of containing one or more thyroid hormones, removing proteins from the sample, separating the thyroid hormone from the sample, ionizing the thyroid hormones, for example by electrospray ionization, and analyzing the hormone in a mass spectrometer, preferably in the negative mode.

[00028] Accordingly, there is also provided a method for the simultaneous or sequential analysis of one or more thyroid hormones comprising: obtaining a sample containing or suspected of containing one or more thyroid hormones, removing proteins from the sample, separating the thyroid hormones from the sample, ionizing the thyroid hormone, for example by electrospray ionization, and analyzing the hormones in a mass spectrometer, preferably in the negative mode.

[00029] Accordingly, there is also provided a method for the simultaneous or sequential analysis of a plurality of thyroid hormones and a plurality of steroid hormones comprising: obtaining a sample containing or suspected of containing a plurality of hormones, removing proteins from the sample, separating the hormones from the sample, ionizing the thyroid hormones, for example, by electrospray ionization, ionizing the steroid hormones by photoionization, and analyzing the hormones in a mass spectrometer, in the negative or positive modes.

Brief Description of the Drawings

[00030] The invention, including the best approaches known to the inventors, can be better understood with reference to the following detailed description taken in combination with the following drawings, in which:

[00031] Figure 1 is a mass spectrum of a sample of plasma containing T4 and T3.

[00032] Figure 2 is a mass spectrum of a globulin standard containing T4 and T3.

[00033] Figure 3 is a typical tandem mass spectrometric chromatogram obtained for T4 and T3.

[00034] Figure 4 is a graph showing T3 measured by Isotope Dilution Tandem Mass Spectrometry vs. Immunoassay.

[00035] Figure 5 is a graph showing T4 measured by Isotope Dilution Tandem Mass Spectrometry vs. Immunoassay.

Detailed Description of the Exemplified Embodiment

[00036] The invention provides methods of analysis for hormones. The hormones may include:

Dehydroepiandrosterone (DHEA)
Dehydroepiandrosterone sulphate (DHEAS)
Aldosterone
Cortisol
11-Deoxycortisol
Androstenedione
Testosterone
Estradiol
17-OH Progesterone
Progesterone
Allopregnanolone
16-OH Estrone
2-OH Estrone
Estrone
Estriol
Vitamin D
thyroxine
free thyroxine
triiodothyronine
catecholamines

metanephrines
other steroid hormones
other thyroid hormones
other small peptide hormones
other amines

Sample

[00037] Any sample containing or suspected of containing a hormone can be used, including a sample of blood, plasma, serum, urine or saliva. The sample may contain both free and conjugated or bound hormones. A sample size of at least about 100 μL for hormones generally, or at least about 700 μL for steroid hormones, is presently preferred.

Deproteinization

[00038] The sample is de-proteinated. This can be done by conventional techniques known to those skilled in the art. For example, a sample can be de-proteinated with acetonitrile, containing internal standard, followed by vortexing and centrifugation. The internal standard may be, for example, the deuterated hormone.

Separation of hormones from the sample

[00039] The hormones are separated by methods known to those skilled in the art. For example, the hormones may be separated by liquid chromatography through a column. The column may be a C-18 column. The hormones are subsequently eluted from the column.

Introduction of hormones into a mass spectrometer

[00040] The hormones are then introduced into a mass spectrometer. Optionally, the separation step and step of introducing the hormones into a mass spectrometer can be combined using a combined liquid chromatography spectrometry apparatus (LC/MS). This procedure is based on an online extraction of the injected sample with subsequent introduction into the mass spectrometer using a built-in switching valve.

Isotope Dilution Tandem Mass Spectrometry

[00041] Isotope dilution tandem mass spectrometry incorporates additional dilution steps that act as an internal calibration so that an independent isotopic reference material is not required. It avoids the need to measure the isotope ratio of the highly enriched spike directly, and enables the final results to be arranged as a combination of measurements that are largely insensitive to instrumental bias and drift. Consequently, it has the potential to extend the scope of application of isotope dilution tandem mass spectrometry to include analysis for which reference materials with certified isotope ratios are not available or where contamination of the instrument by the highly-enriched spike causes difficulty.

Instrumentation and ionization techniques

[00042] The hormones are subjected to ionization. Various ionization techniques can be used. For example, photoionization, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and electron capture ionization may be used. Preferably, electrospray ionization is utilized when analyzing thyroid hormones.

[00043] The following mass spectrometers can be used: any tandem-mass spectrometer, including hybrid quadrupole-linear ion trap mass spectrometers and liquid chromatography-tandem mass spectrometers such as the API 2000™ mass spectrometer, the API 3000™ mass spectrometer, and the API 4000™ mass spectrometer, described in U.S. patents 4,121,099; 4,137,750; 4,328,420; 4,963,736; 5,179,278; 5,248,875; 5,412,208; and 5,847,386 (Applied Biosystems/MDS SCIEX, Foster City, Calif./Concord Ontario, Canada). When analyzing thyroid hormones, a spectrometer with a turbo spray ion source, such as the API 2000™ and API 3000™ mass spectrometers, is presently preferred. When analyzing FT4, the API 4000™ mass spectrometer is presently preferred.

[00044] Ionization may be performed by utilizing the mass spectrometer in the negative or the positive mode, depending on a particular analyte's tendency to give rise to a particular ion form, as is known to those skilled in the art. Typically, for thyroid hormones, the spectrometer is employed in the negative mode.

[00045] Hormones are identified on the basis of the mass to charge ratio of their molecular ions and fragment ions, as is known to those skilled in the art. When the hormones are purified by liquid chromatography, they can also be identified by their retention times.

[00046] Hormones are quantified by their intensity as determined in the mass spectrometer in counts per second. Calibration curves for known concentrations of the hormones are established for comparison.

EXAMPLES

[00047] The invention will now be demonstrated using the following examples, provided to demonstrate but not limit the embodiments of the present invention:

1. Analysis of a sample for thyroid hormones

[00048] A sample of 100 μL of plasma was used. Proteins were precipitated with 150 μL of acetonitrile, capped and vortexed. The sample was then centrifuged, and 200 μL of the supernatant was injected onto a Supelco LC-18-DB™ chromatographic column equipped with Supelco Discovery C-18™ guard column, coupled to a tandem mass spectrometer (LC/MS/MS). The column was washed with 20% methanol in 5mM ammonium acetate for 3 minutes. The valve was switched and the sample was eluted in 75% to 95% methanol. The total run time was 6 minutes. Slight adjustments to the volumes, concentrations and times described can be made, as is known to those skilled in the art.

[00049] The eluant was introduced into an ion-spray ionization chamber and analyzed by API 2000™ mass spectrometer using the negative mode. The mass/charge ratios for T4 and T3 ions is 775.8 and 650 respectively. The ionization may be by electrospray using a turboionspray chamber.

[00050] This demonstrates a simple method of preparing a complex biological matrix for analysis of hormone content, and a sensitive analytical method that permits the simultaneous analysis of two hormones, T3 and T4.

2. Analysis of thyroid hormones using a methanol gradient to elute the hormones

[00051] A sample of 100 μL of plasma was used. Proteins were precipitated with 150 μL of acetonitrile, containing an internal standard of deuterated T_4, and vortexed. The sample was centrifuged, and 200 μL of the supernatant was injected onto a C-18 column coupled to a tandem mass spectrometer (LC/MS/MS). The column was washed with 20% methanol in 5mM ammonium acetate for 3 minutes. The valve on the column was switched and the sample was eluted in a methanol gradient of 20 to 100%. The total run time was 7 minutes. Slight adjustments to the volumes, concentrations and times described can be made by those skilled in the art.

[00052] A sample of the eluant was introduced into an ion-spray ionization chamber and analyzed by an AP 2000™ mass spectrometer using the negative mode. The ionization may be by electrospray using a turbolonspray chamber. See Figure 1 and Figure 2 for mass spectrums generated for T3 and T4.

[00053] This demonstrates a simple method of preparing a complex biological matrix for analysis of thyroid hormone content, and a sensitive analytical method that permits the simultaneous analysis of multiple hormones.

3. Analysis of thyroid hormones using isotope dilution tandem mass spectrometry

[00054] This example describes an isotope dilution tandem mass spectrometry method for the simultaneous determination of T4 and T3 in serum. The method is accurate, specific, precise (%CVs between 3.5 and 9.0), simple - requiring no extraction and only protein precipitation and fast (<7 min).

Chemicals and reagents

[00055] Standards of T4 and T3 were purchased from Sigma (St. Louis, MO, USA). A stable deuterium-labeled internal standard, L-thyroxin-d_2 was synthesized according to procedures described in the literature [16], [17] by Dr Tomas Class from the Chemistry Department at Georgetown University. HPLC grade methanol was purchased from VWR Scientific. All other chemicals were of analytical grade and purchased from Sigma.

Solutions and standards

[00056] Stock solutions of T3, T4 and internal standard (IS) were prepared separately to obtain concentration of 1mg/mL for each. 40% ammonium hydroxide (v/v) in methanol was used as a solvent. The analyte stock solutions were diluted with methanol to obtain the spiking solutions. The solutions were stored at 4°C and could be used for several months. Standards for the calibration curve in the range of 0.325 to 5 ng/mL for T3 and 12.5 to 200 ng/mL for T4 were prepared by adding the analyses to 3 % human γ-globulin (volume of spiking solution < 2% of final volume). Quality control (QC) samples (Diagnostic Product Corp., Los Angeles, USA) at low, medium and high levels were used. A solution of 50-ng/mL d_2-T4 in methanol was used as the internal standard.

Sample preparation

[00057] Serum/plasma samples were thawed at room temperature. 150 µL of IS solution was added to aliquots of 100 µL of the serum or plasma sample. After 30 seconds of vortex mixing, the samples were stored for 10 min at room temperature to allow complete protein precipitation. The samples were centrifuged for 10 min at 15,000 rpm and 100 µl of supernatant was injected into the LC-MS-MS system.

LC/MS/MS conditions.

[00058] An API 3000™ tandem mass-spectrometer (SCIEX, Toronto, Canada) equipped with TurbolonSpray and Shimadzu HPLC system was used to perform the analysis. Negative ion multiple reaction-monitoring (MRM) mode was used. The transitions to monitor were selected at m/z 650→ 127 for T3, m/z 776→ 127 for T4, m/z 778→ 127 for d_2-T4. Nitrogen served as auxiliary, curtain and collision gas. Gas flow rates, source temperature, Ion Spray voltages and collision energies were optimized for every compound by infusion of 1µg/mL of the standard solutions in methanol at 20 µL/min and by flow-injection analysis (FIA) at LC flow rate. The main working parameters for the mass spectrometer are summarized in Table 3. Data processing was performed on Analyst 1.2 software package.

LC-MS-MS procedure

[00059] The procedure is based on an online extraction/cleaning of the injected samples with subsequent introduction into the mass-spectrometer by using a built-in Valco switching valve. 100 µl of the sample were injected onto a Supelco LC-18-DB (3.3 cm x 3.0 mm, 3.0 µm ID) chromatographic column equipped with a Supelco Discovery C-18 (3.0 mm) Guard column, where it underwent cleaning with 20% (v/v) methanol in 5 mM ammonium acetate pH=4.0 at flow rate 0.8 mL/min. After 3.5 min of cleaning the switching valve was activated, the column was flushed with water/ methanol gradient at flow rate 0.5 mL/min and the samples were introduced into the mass-spectrometer. The gradient parameters are shown in Table 4.

Immunoassays for T4 and T3

[0060] T4 was measured by the Dade RxL Dimension (Dade–Behring Diagnostics, Glasgow, DE) and T3 by the DPC Immulite (Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's specifications.

Results

[00060] The optimal mass spectrometer working parameters are shown in Tables 3 and 4.

[00061] Replicate sera were assayed both within-day and between-day at several concentrations. The within-day and between-day precision data is provided in Tables 5 and 6.

[00062] Recovery studies for T4 and T3 are shown in Tables 7 and 8. All results shown are the means of 8 replicates.

[00063] Figure 3 shows a typical tandem mass spectrometric chromatogram obtained for T3 and T4.

[00064] Specimens were tested for T3 and T4 by both immunoassay (T3 DPC Immulite, T4 Dade Behring Dimension RxL) and by tandem mass spectrometry. Linear regression correlations (Prism) are shown in Figures 4 and 5.

[00065] The lower limit of quantitation of the mass spectrometry method was found to be 0.15 ng/mL for both T3 and T4. Detection limit was around 0.062 ng/mL.

Discussion

[00066] Evidence initially gleaned from both the CAP PT Program and pediatric reference ranges employing different immunoassays indicated the probability of lack of specificity for T4 and T3 immunoassay tests. To adequately assess this phenomenon we developed the isotope dilution tandem mass spectrometric method described in this example. Serum T4 and T3 detection methods have evolved through a variety of technologies since the 1950s. Radioimmunoassay (RIA) methods to detect THs were developed in the 1970s. Serum T4 and T3 concentrations are currently measured by competitive immunoassay methods (IAs) that are mostly non-isotopic and use enzymes, fluorescence or chemiluminescence molecules as signals [27]. Table 2 clearly indicates that current IAs for T4 and T3 lack specificity and give mean results differing by a factor of approximately 2 in CAP PT programs. Total hormone assays necessitate the inclusion of a displacing agent (such as salicylate) to release the hormone from its binding proteins [28]. The displacement of hormone binding from serum proteins by such agents, together with the large sample dilution employed in modern assays, facilitates the binding of hormone to the antibody reagent.

[00067] Since T3 is ten-fold lower in concentration compared with T4 in blood it therefore presents both a technical sensitivity and precision challenge despite the use of a higher specimen volume. Although a reliable high-range T3 measurement is critical for diagnosing hyperthyroidism, a reliable normal-range measurement is also important for adjusting antithyroid drug dosage and detecting hyperthyroidism in sick hospitalized patients, in whom a paradoxically normal T3 value may indicate hyperthyroidism.

[00068] The correlation coefficient for the T4 comparisons (0.931) is significantly better than for the T3 comparisons (0.848) (Figures 4 and 5). T3 by tandem mass

spectrometry gave slightly higher results than those obtained by the DPC Immulite (Figure 4). While this is true for children, our preliminary data for non-pregnant and pregnant women indicates a very poor correlation for T3 in both groups (r between 0.407-0.574).

[00069] The reasons for this are not clear but could include standardization issues, heterophilic antibodies etc. Of importance, we determined that reverse T3, which lacks a daughter ion of 127 m/z, therefore does not interfere in our tandem mass spectrometry method. Applying the tandem mass spectrometric method to CAP PT samples in the K/KN general ligand program again revealed that around 85% of the immunoassay methods gave means on samples which were lower than the means obtained by our tandem mass spectrometry method while 15% had higher means.

[00070] In conclusion, correlations between immunoassays and tandem mass spectrometry for T4 proved to be adequate except for the pregnant population, while the data for T3 was far less impressive especially during pregnancy. Recovery studies from several different sera using deuterated T4 as internal standard showed consistent (90-109%) recoveries for both T4 and T3 (Tables 7 and 8). The recovery differences found between samples were surprisingly larger for T4 than for T3. This indicates a lack of need to use deuterated T3 as the T3 internal standard. The isotope dilution tandem mass spectrometric method we developed is rapid (<7 min), accurate (provides the true result as has been assessed by recovery studies), specific (measures only the analyte it purports to measure), precise (low %CV) and easy to perform.

Parameter	Value
Nebulizer gas (NEB)	8
Curtain gas (CUR)	10
Collision gas (CAD)	6
Turbolon Spray Heater gas	7 L/min
Turbolon Spray (IS) voltage	4500 V
Entrance Potential (EP)	7.5 V
Collision cell Exit Potential (CXP)	5 V
Source temperature	450°
Dwell time	250 msec

Table 3: Tandem mass-spectrometer working parameters

Time (min)	Methanol (%)
3.50	75
5.25	76
5.50	100
7.00	End

Table 4: Gradient parameters

Analyte	CONTROL 1			CONTROL 2		
	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)
T3	1.04	0.014	1.36	2.44	0.077	3.19
T4	24.1	0.437	1.81	81.2	1.502	1.85

Table 5: Within day precision (n=10)

Analyte	CONTROL 1			CONTROL 2			CONTROL 3		
	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)
T3	1.08	0.05	4.47	2.39	0.22	9.21	3.49	0.31	9.00
T4	24.4	1.39	5.69	76.6	3.11	4.06	116.3	4.15	3.57

Table 6: Between day precision (n=20, 1 run per day for 20 days)

Sample #	Added (ng/mL)	Detected mean	Added amount recovered	Recovery, %
1 (n=8)	0	85.9	NA*	NA
	10	96.7	10.8	108.0
	40	127.5	41.6	104.0
2 (n=5)	0	62.6	NA	NA
	10	72.1	9.5	95.0
	40	98.0	35.4	90.0
3 (n=5)	0	73.8	NA	NA
	10	84.7	10.9	109.0
	40	116	42.2	105.0
4 (n=5)	0	58.3	NA	NA
	10	68.0	9.7	97.0
	40	95.0	36.7	92.0

*NA - not applicable

Table 7: Recovery of added thyroxine (T4)

Sample #	Added (ng/mL)	Detected mean	Added amount recovered	Recovery, %
1 (n=8)	0	1.88	NA	NA
	0.25	2.12	0.24	96.0
	1.00	2.85	0.97	97.0
2 (n=5)	0	1.70	NA	NA
	0.25	1.96	0.26	104.0
	1.00	2.76	1.06	106.0
3 (n=5)	0	1.56	NA	NA
	0.25	1.81	0.25	100.0
	1.00	2.62	1.06	106.0
4 (n=5)	0	0.49	NA	NA
	0.25	0.74	0.25	100.0
	1.00	1.50	1.01	101.0

*NA - not applicable

Table 8: Recovery of added triiodothyronine (T3)

4. Analysis of thyroid hormones and steroid hormones

[00071] A sample of 100 μL of plasma is used. Proteins are precipitated with 150 μL of acetonitrile and vortexed. The sample is centrifuged, and 200 μL of the supernatant is injected onto a C-18 column coupled to a tandem mass spectrometer (LC/MS/MS). The column is washed with 20% methanol in 5mM ammonium acetate for 3 minutes. The valve on the column is switched and the sample is eluted in a methanol gradient of 20 to 100%. The total run time is 10 minutes. Slight adjustments to the volumes, concentrations and times described can be made, as is known to those skilled in the art.

[00072] A sample of the eluant is introduced into an ion-spray ionization chamber and analyzed by API 3000™ mass spectrometer using the negative mode for thyroid hormones in the sample. Steroid hormones in the sample are ionized by photoionization, with the spectrometer in the negative or positive mode. Analysis in the positive mode is typically made for DHEA, Aldosterone, Cortisol, 11-Deoxycortisol, Androstenedione, Testosterone, Estradiol, 17-OH Progesterone, Progesterone, Allopregnalone, and Vitamin D, whereas analysis in the negative mode is typically made for 16-OH Estrone, 2-OH Estrone, Estriol and DHEAS. However, it is possible to analyze any of the hormones in either positive or negative mode.

[00073] This demonstrates a simple method of preparing a complex biological matrix for analysis of possible steroid and thyroid hormone content, and a sensitive analytical method that permits the simultaneous analysis of steroid and thyroid hormones.

[00074] The results indicate that this technique, allows for the identification and characterization of low levels of thyroid hormone in human plasma and saliva.

[00075] While the above detailed description describes the exemplifying embodiments of the present invention, it should be understood that the present invention is susceptible to modifications, variations and alterations without deviating from the scope of the invention.

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